South Carolina
Environmental Laboratory Certification Criteria and Methodology: Biological Parameters

South Carolina Department of Health and Environmental Control
Bureau of Environmental Services
Office of Environmental Laboratory Certification

October 1996

DISCLAIMER

This document reflects the current acceptable South Carolina Department of Health and Environmental Control (SCDHEC) and U. S. Environmental Protection Agency (USEPA) methodologies, as of August 1996. Any revisions in SCDHEC and/or USEPA methodologies after this date take precedence over those specified in this document. The Office of Environmental Laboratory Certification will notify laboratories of major changes that may affect their procedures or certification status.

Mention of trade names or commercial products does not constitute endorsement by the SCDHEC. In instances when single source vendors have been specified due to quality assurance considerations, the laboratory will be allowed to use an alternative product after demonstrating equivalency to the specified product.

A laboratory may request in writing a variance to the requirements presented in this document. The laboratory must demonstrate with appropriate documentation that the requested change fulfills the objective(s) of the original requirement.

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1. Introduction

On April 9, 1980, the "State Environmental Laboratory Certification Regulation 61-81" was passed by the South Carolina Legislature. The regulation provided the mechanism whereby the South Carolina Department of Health and Environmental Control (SCDHEC) certifies all state, federal, municipal, field, and commercial laboratories that submit environmental data to the Department. The Office of Environmental Laboratory Certification, hereafter referred to as the Office, was created to assume this responsibility. Laboratories are evaluated and certified to assure that laboratory data submitted to the SCDHEC will be scientifically valid and legally defensible, and of known and acceptable precision, accuracy and integrity.

This manual addresses the biological parameters for which certification is available. It informs applicants requesting certification for biological parameters of the standards that they must meet so their data will be accepted by the SCDHEC for compliance with the National Pollutant Discharge Elimination System (NPDES) Permits. Criteria are based on the EPA s Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms, (EPA/600/4-90/027F), the Short-Term Methods For Estimating The Chronic Toxicity Of Effluents And Receiving Water To Freshwater Organisms, (EPA-600-4-91-002) and the Short-Term Methods for Estimating the Chronic Toxicity Of Effluents And Receiving Water To Marine And Estuarine Organisms, (EPA-600-4-91-003). The South Carolina Procedures for the Pass/Fail Modifications of the *Ceriodaphnia* 48-Hour Acute Toxicity Test and *Ceriodaphnia* Survival and Reproduction Test, 1989 have been replaced by the "Statistical Analyses" section of this document. The EPA manuals were promulgated in the 40 CFR Part 136 Whole Effluent Toxicity: Guidelines Establishing Test Procedures for the Analysis of Pollutants, Monday, October 16, 1995 and became effective on November 15, 1995. This manual also informs applicants of the certification process.

Regulation 61-9, Water Pollution Control Permits, page 72, paragraph five (5) states The Clean Water Act provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under this permit shall, upon conviction, be punished by a fine of not more than $10,000 or by imprisonment for not more than 2 years, or both. If a conviction of a person is for a violation committed after a first conviction of such person under this paragraph, punishment is a fine of not more than $20,000 per day of violation, or by imprisonment of not more than 4 years, or both. Falsifying, tampering with or rendering inaccurate any test, including the early termination of an otherwise valid toxicity test, is not allowed. It may also include other unapproved deviations from protocol.

B. Application Process

1. Application
   a) This Office will send to the laboratory, at the laboratory s request, an application package which will include an application form, a fee assessment schedule, a copy of Regulation 61-81, instructions for filing an application, reference materials list, self-check list, list of NIST approved proficiency testing (PT) providers, and a cover letter. Additional information, such as guidance documents or criteria manuals, may be sent with the application if the parameters for which the laboratory is seeking certification are known.

   b) The completed application form and supplemental materials, along with the application fee, must be submitted to this Office together. When an application package is received, it is reviewed for completeness. All incomplete applications will be returned to the applicant with a letter listing the deficiencies. The laboratory will have to resubmit the materials for the certification process to continue.

2. Application Review
   a) Once all components of the application have been received, the materials will undergo a thorough technical review. Additional clarification, documentation, or procedural modifications may be requested from the applicant.

3. On-site Evaluation – Instate Laboratories
   a) Laboratories located in the state of South Carolina will participate in an on-site evaluation to ascertain the laboratory s ability to produce valid data. Facilities, instrumentation, equipment, personnel, sample handling, quality control, and analytical records will be reviewed. Deficiencies and deviations, if present, will be addressed during the evaluation. A narrative report listing these deficiencies will be sent to the laboratory within thirty days. The laboratory will have up to thirty days after receipt of this report to correct the deficiencies. Once all deficiencies have been corrected, certification can be granted. The certification period of up to three years will be granted. To renew certification, the evaluation process will be repeated.

4. On-site Evaluations – Out-of-State Laboratories
   a) Laboratories located outside of the state of South Carolina must have participated in an on-site evaluation conducted by an approved state certifying authority. A copy of the most recent certificate, parameter list, on-site evaluation report, results of performance evaluation studies and the laboratory s response to this report must be submitted for review.

   b) The evaluation report must specifically address those parameters for which certification in South Carolina has been requested. The certificate and parameter list should state the type of tests and the species used. The evaluation of the laboratory s capabilities to perform additional required parameters (pH, dissolved oxygen, residual chlorine, alkalinity, hardness and conductivity) must be addressed

in the on-site evaluation report if not specifically listed on the certificate and/or parameter list.

Both in-state and out-of-state laboratories will have to submit a completed self-check list obtained from this Office with the application.

5. Application Fees

An application fee must be submitted with each application to cover processing. Certification fees are paid when the laboratory becomes certified. Certification fees are not pro-rated. If the laboratory is certified for any portion of a fiscal year, the entire certification fee must be paid. The fiscal year runs from July 1 to June 30.

C. Parameters

This Office offers certification to qualified laboratories which have had an on-site evaluation by an approved certifying authority, and have passed performance evaluation samples for all pertinent parameters.

Applicants may be certified for the following biological parameters using only the approved method references listed:

PARAMETER	REFERENCE
1. Toxicity	
A. Acute - Ceriodaphnia dubia[1]	EPA 1002.0, EPA/600/4-90/027F[2], 1993
B. Chronic - Ceriodaphnia dubia[1]	EPA 1002.0, EPA-600-4-91-002[3], 1994
C. Acute - Mysidopsis bahia	EPA 1007.0, EPA/600/4-90/027F[2], 1993
D. Chronic - Mysidopsis bahia	EPA 1007.0,EPA-600-4-91-003[4], 1994
2. Taxonomic Identifications	

[1]Laboratories certified for toxicity testing must also be certified for hydrogen-ion concentration (pH), dissolved oxygen, conductivity, total hardness, alkalinity, total residual chlorine, and total dissolved solids using approved methodology as specified in the Federal Register, 40 CFR Part 136, April 4, 1995.

[2]"Methods for Measuring the Acute Toxicity of Effluents and Receiving Waters to Freshwater and Marine Organisms", Fourth Edition, August 1993.

[3]"Short-Term Methods for Estimating the Chronic Toxicity of Effluents and Receiving Waters to Freshwater Organisms", Third Edition, July 1994.

[4]"Short-Term Methods for Estimating the Chronic Toxicity of Effluent and Receiving Waters to Marine and Estuarine Organisms," Second Edition, July 1994.

A. Phytoplankton		Note[5]
B. Freshwater Zooplankton	Note[5]	
C. Freshwater Macroinvertebrates		Note[5]
D. Estuarine/Marine Macroinvertebrates	Note[5]	
E. Freshwater Fish	Note[5]	
F. Estuarine/Marine Fish		Note[5]
G. Ichthyoplankton	Note[5]	
H. Periphyton	Note[5]	
I. Macrophytes	Note[5]	
3. Microbiology		
A. Microscopic Particulate		EPA 910/9-92-029, 1992

[5]For taxonomic identifications, the laboratory must demonstrate access to a suitable reference or type specimen collection and possess adequate reference materials and taxonomic keys.

Analysis[6]

4.	Biological Examinations	
	A. Biomass	SM18: 10200
	B. Biomass, Periphyton	SM18: 10300D
	C. Chlorophyll a, Phytoplankton	SM18: 10200H
	D. Chlorophyll a, Periphyton	SM18: 10300C

A laboratory can request certification for additional parameters if the data will be submitted to SCDHEC for compliance or monitoring purposes. Parameters other than those listed above may be required by an NPDES permit. In these cases, the permit will reference specific documents or methods, or the NPDES permit may contain the methodology.

[6]Certification for this parameter does not include identification of Giardia or Cryptosporidium.

4. General Criteria

A. Personnel

A laboratory certified for effluent toxicity testing must have on staff at least one full-time employee with a bachelor of science degree in the biological sciences (or a closely related field), and three years of experience in toxicity testing. This person must have the primary responsibility for conducting the toxicity tests. A graduate degree in environmental toxicology may be substituted for three years of experience. A graduate degree which includes exposure to toxicological tests may be substituted for two years of experience.

A laboratory certified for taxonomy must have on staff an employee with a bachelor of science degree in the biological sciences and two years experience in taxonomy. This person must perform the taxonomic identifications reported by the laboratory. A graduate degree in biology which includes taxonomic identifications may be substituted for two years of experience.

A laboratory certified for microscopic particulate analysis must have on staff an employee with a bachelor of science degree in the biological sciences and two years of experience in microscopic particulate analysis. This person must perform the actual analysis and must perform or oversee the sampling process. A graduate degree in biology with exposure to this type of analysis may be substituted for two years of experience.

A laboratory certified for biological assays (biomass, chlorophyll a) must have on staff an employee with a bachelor of science degree in the biological sciences and at least three years of experience in performing bioassays or related analyses. A graduate degree in biology with exposure to this type of analysis may be substituted for two years of experience.

The laboratory must notify this Office in writing of any changes in the laboratory name, ownership or location, and in laboratory supervision or analysts directly involved with the approved parameters. These notices must be submitted within seven (7) days of the change.

B. References

The laboratory must have available on site copies of the documents and manuals necessary to perform the certified parameters. Approved methodology for toxicity testing and inorganic parameters are listed in the Federal Register, 40 CFR Part 136, Whole Effluent Toxicity: Guidelines Establishing Test Procedures for the Analysis of Pollutants, October 16, 1995 and the Federal Register, 40 CFR Part 136, Guidelines Establishing Test Procedures for the Analysis of Pollutants Under the Clean Water Act, Technical Amendments; Final Rule, April 4, 1995, respectively. Additionally, this document and the South Carolina Procedures for the Pass/Fail Modifications of the *Ceriodaphnia* 48 Hour Acute Toxicity Test and *Ceriodaphnia* Survival and Reproduction Test must be available. It is strongly recommended that commercial laboratories have on hand a copy of the client s NPDES permit describing the toxicity test required. Method references for taxonomic identifications and other bioassays (chlorophyll a and biomass determinations) have been approved by our Office in conjunction with the Water Quality Monitoring Section. The laboratory must request in writing the use of methods other than those listed in Section 3 of this document, or modifications made to the methods listed, and this Office must approve those requests before the laboratory incorporates the variances into its routine.

Additional reference materials may become available. This Office will notify all relevant certified laboratories of the availability of these materials.

C. Standard Operating Procedures (SOP) Manual

The laboratory must have a Standard Operating Procedures document (SOP) available to all laboratory personnel. The SOP must address, but not be limited to, the following items:

1. Organization and management of the laboratory.

2. Sample collection, labeling, preservation, tracking (chain-of-custody), and handling (holding times, storage, and disposal) of samples.

3. Preparation, labeling, storage conditions, and shelf-life of all solutions used.

4. A list of all instruments and equipment required for the analyses; procedures for the calibration, use, and maintenance of equipment and instruments; and criteria for acceptable instrument performance.

5. Analytical and biological methodology necessary to perform each test.

6. Quality assurance/quality control procedures.

7. Care, use, and maintenance of test organisms.

8. Explanation of record keeping and documentation. Documentation must allow the reconstruction of all actions taken on the sample from the time of sample collection through to its analysis.

9. An explanation of the statistical analyses or data reduction methods used.

All parameters for which the laboratory has requested certification in the application must be covered in the SOP document. The presentation should be simple and complete so that a user with a basic undergraduate science education and experience could follow through the analysis and data handling. The procedures must conform to current SC DHEC and U. S. EPA regulations. If a procedure is referenced, the reference must be specific (i.e. method number, date published, etc.) and the presentation of the method must be clear. Instrument installation and use must agree with manufacturer's instructions. Deviations must be approved by this Office and documented.

D. Facilities

Laboratory space must be adequate to accommodate periods of peak work load. Facilities must be clean, safe, secure, well-ventilated, and environmentally controlled to maintain a temperature of approximately 78 F, and be adequately lighted (10 - 20 ì E/M^2/5 or 50 - 100 ft-c) at the bench top. Hot and cold running water must be available.

Space must be available to culture and test the organisms under the recommended conditions within ranges specified in this document.

The space in which test animals are cultured must be separate from the space in which the tests

are performed. If upright incubators are used, at least two must be available. One must be used exclusively for cultures, while the other must be used exclusively for tests. If the laboratory is equipped with a walk-in incubator, cultures and tests must be conducted in different areas. Under no circumstance should test boards be stored on shelves above, below, or near culture boards. The walk-in incubator should have an independent air system, and air flow from the tests should not flow near or across the cultures in the incubator. The ventilation system for the toxicity laboratory must be separate from the ventilation system serving other areas of the laboratory facility to avoid potentially hazardous vapors from entering the toxicity laboratory.

The area in which tests are initiated and renewed must be temperature stable.

The laboratory must own or be in possession of the equipment necessary to perform the certified parameters. Leased equipment must be accompanied by a written contract covering the certification period. The equipment must be in good working condition and must be available for inspection upon request. Maintenance and repair records should be available for all equipment and instrumentation. These records should include the date, technician, type of maintenance or repair, and date when returned to service.

All chemicals must be at least reagent grade and must be stored according to manufacturer's suggestions. The manufacturer's seal must be broken in the laboratory. The chemical container must be marked with the date it was received, the date it was opened, the initials of the analyst who opened it, and the expiration date. If the manufacturer does not provide an expiration date, one must be assigned to the chemical by the laboratory. Usually, the shelf-life of a chemical should not exceed one year. It is strongly suggested that the laboratory order smaller quantities of reagents to minimize shelf storage.

All solutions must be clearly labeled as to identity, date prepared, the initials of the analysts who prepared it, and the expiration date.

E. Sample Collection and Handling

All samples must be collected by someone instructed on how to properly collect the sample. Approved sample containers which will not affect the integrity of the sample must be used. The sample container should be rinsed with a portion of the sample before filling. The container should be filled and capped to avoid head space. Collections must adhere to EPA protocol.

Sample containers must be made of non-toxic materials. A toxicity test must be performed on each type of container used. A toxicity test must be performed using culture water left in a container for 96 hours. If this solution is determined to be toxic, this type of container must not be used. Also, the laboratory should regularly repeat this test to assure that changes in the manufacturing process or other outside influence has not rendered the containers toxic. In addition, the test must be repeated when a new vendor or sample container is used. Documentation of these tests must be available for examination.

Each laboratory must have a written procedure for sample handling in its standard operating procedures manual. The laboratory is required to maintain accurate written records tracing the possession and transfer of samples from collection to analysis for all regulatory samples. Sample transfer may be handled by the client, by laboratory personnel, or by commercial courier. A composite sample must be maintained at 4^{o} C during the 24 1 hour compositing period. The sample must be refrigerated until analysis has begun. If more than two hours will elapse between the time a grab sample is collected and test initiation, the grab sample must be cooled to 1 - 4^{o} C immediately after it is collected and samples must remain at 1 - 4^{o} C during shipping and storage. Wet ice is the best material to use to ensure the sample arrives at the desired temperature.

Custody of the sample must be clearly documented. It must be uninterrupted from the time the sample was taken, through the time it was received by the laboratory and analyzed.

The chain of custody information must include, but is not limited to, the information below.

1. Sample identification number

2. Location of sampling (facility and outfall number)

3. Client/Facility and NPDES number

4. Sample collector s signature.

5. Sample type (grab or composite).

6. Type of analyses required.

7. Date and time collected. Composite samples must have the starting and ending times and dates for the composite period documented and must be iced during collection.

8. Preservatives used. Note: Ice is considered a preservative and if the sample is shipped on ice, it should be documented.

9. Program area (drinking water, waste water, ground water, etc.)

10. Sample matrix (liquid, soil, etc.)

11. General description of the sample (odor, color, turbidity, foam, etc.)

12. Transfer signatures with the dates and times for both the person relinquishing the sample and the person receiving the sample for all transfers. If the sample is shipped via commercial courier, this should be noted on the chain of custody form by the facility before shipping and by the laboratory upon receipt of the samples.

13. Comments to include any additional information, such as weather conditions, field parameter information, etc.

14. The shipping receipts should be attached to the chain of custody forms and maintained as part of the permanent record.

The laboratory should maintain a written log of all samples received. This should include the date and time received, person receiving, sample identification, analyses required, number of containers, receipt temperature, and comments.

The laboratory performing the analyses must determine whether or not the sample is invalid. It is the laboratory s responsibility to inform the client of any deficiencies noticed with the sample upon receipt. Examples of deficiencies include ruptured containers or receipt of samples not shipped on ice.

When its clients collect the samples, the laboratory must inform them of the required sample collection procedures and chain-of-custody documentation. When these requirements have not been met but the sample is used to perform a test, the report to SC DHEC containing the results must be accompanied by a statement of the deficiencies. If the laboratory does not report the data to SC DHEC directly, then it must inform the client of this requirement and provide the client with a statement of the deficiencies. The laboratory's protocol for handling data that are generated using invalid samples must be included in the SOP document.

All samples must be collected as described in the NPDES permit. Laboratories not collecting samples should verify that clients are collecting samples properly.

The laboratory must determine the temperature of a sample when it is received. This may be done directly on an aliquot of the sample or indirectly by measuring the temperature of a temperature control bottle or the melted ice in the shipping container. Receipt temperature must be documented. Laboratories should notify clients of sample temperatures which are greater than 8 C.

The sample label must be moisture resistant and must be securely attached to the sample container. The label must include the following information:

1. Unique sample identification
2. Location at which sample was taken
3. Collector's initials or signature
4. Type of test required
5. Date and time of collection
6. Type of sample

The holding time is the number of hours that have elapsed from the time the sample was collected to the first use of sample at test initiation. For composite samples, the holding time starts at the end of the compositing period. This is the time that the last aliquot of sample was collected, which is not necessarily the same time that the sample is harvested from the composit samples.

For acute toxicity tests, the holding time of the sample must not exceed 36 hours. An acute test must not be performed using more than one sample.

At least three samples must be collected for chronic toxicity tests. Two of these samples must be used to renew test solutions on no more than two consecutive days. One of the samples must be used to renew test solutions on no more than three consecutive days. The holding time for each sample must not exceed 36 hours, and under no circumstance can the sample be used after 80 hours.

After receipt into the laboratory, a sample must be stored at 1 - 4^{o} C. Samples must not be allowed to freeze. If a sample contains ice crystals or is frozen, it must not be used for analysis.

F. Documentation

The laboratory must retain all calibration, analytical, chain of custody, and quality control records and all other documentation relevant to sample analyses for at least five years or longer if required by regulation. Records must be written in indelible ink. The use of pencils is not allowed. Corrections are make by striking through the error once. The analyst then writes the date, his/her initials, and the correction. If necessary, an explanation why the correction was made should be included.

Documentation should be in such detail that it is legally defensible. The information documented should be clear and well organized. All columns and rows must have headings describing the information contained within. The units of measurements must be clearly stated for all quantitative data. The information documented should represent the activity in enough detail so that the activity could be reconstructed if necessary. All entries must be dated, and the person making the entry must be identified.

The original chain-of-custody forms must be completed (receipt signature, date, time, and other information) and retained by the contract laboratory. Copies of the completed chain-of-custody form should be provided to the permittee if requested.

G. Proficiency Testing

All laboratories certified for toxicity testing are required to participate in the EPA s DMR-QA Performance Evaluation Studies annually and analyze the toxicity samples. If the laboratory receives Not Acceptable results, the laboratory must review and reconstruct the test to determine the cause of the failure. The laboratory s findings must be detailed in writing and must accompany the results. Make-up samples are not available for this parameter.

Laboratories are also required to either participate in the EPA's Water Pollution (WP) Studies or purchase blind audit samples from a commercial vendor annually.

5. Minimum Criteria: Toxicity Testing

The laboratory must be familiar and proficient at the referenced methodology, and must incorporate the requirements of this Office which are addressed in this document. This document supercedes the December 1989 South Carolina Environmental Laboratory Certification Criteria: Biological Parameters and the May 1989 South Carolina Procedures for Screening Modifications of the Ceriodaphnia 48-hour Acute Toxicity Test and Ceriodaphnia Survival and Reproduction Test.

A. Test Organisms

Test organisms must be obtained directly from an approved vendor or from the EPA Newtown facility. Organisms for initiating cultures will be provided.

Requests for organisms can be faxed or mailed to the attention of James M. Lazorchak or the Bioassessment and Ecotoxicology Branch. His fax number is (513)569-7081. The following information must be provided:

Name
Organization
Purpose (Superfund, NPDES, RCRA, etc.)
Number and type of organism needed
Age of organism needed
Date needed
Account number for shipment of organisms

A seven day advance notice is required when ordering Ceriodaphnia dubia.

The source of the organisms must provide a letter or certificate verifying the identity of the organisms. This must be retained by the laboratory.

The culture line used by the laboratory must be initiated with one female only. This specimen must be mounted permanently on a slide. Every six months thereafter, a representative specimen must be sacrificed and mounted permanently on a slide. A mounting medium, such as CMC-10 or CMC-9AB, can be obtained from Master s Chemical Company by calling (708)238-9292. The organism must be identified to species, and the identification must be verified by an additional taxonomist.

The culture s acute and chronic response must be determined before use by establishing a baseline response composed of at least five reference toxicant tests performed on five different generations. A reference toxicant test must be performed on the culture line monthly thereafter. See the "Reference Toxicant Tests" section for additional information.

B. Equipment and Supplies

The following are required equipment and the records that must be maintained on the equipment:

1. Environmental incubator(s): Temperatures must be stable at 25 1 C. Twice-daily temperature readings, taken at least four hours apart, for each of three thermometers evenly spaced throughout

the incubator must be documented. Documentation must include the date, time, analyst, thermometer identification, and temperature reading.

The photoperiod (16 hours light/8 hours dark) must be verified to be correct within 10 minutes twice a year. Photoperiod verifications must be conducted at the normal setting of the incubator and must cover one complete cycle. Whenever the photoperiod times are adjusted, the photoperiod must be verified and documented. Test organisms and cultures must not be kept in the same upright incubator.

2. Refrigerators: Water temperatures must maintain temperature between 1 - 5°C. Temperatures must be recorded twice daily and documented, and readings must be separated by at least four hours. Food for human consumption should not be stored in this refrigerator.

3. Water Purification System: A Millipore Milli-Q or other water purification system which will produce water to specifications equal to American Society for Testing Materials Type I. Type I water degrades when stored. The laboratory should produce this water as needed and use it immediately after processing.

Any fundamental changes in the system, as well as routine maintenance, must be documented. The reagent water must be checked for the parameters below at the stated frequency. If any parameter exceeds the specified limit, the system must be serviced or replaced, or another source which provides water meeting these criteria must be used.

The laboratory must also specify written criteria in the standard operating procedures manual (SOP) that will be used to judge when cartridge replacement is necessary. Documentation that these tests have been performed, along with the results, must be available at the laboratory.

Parameter	Limit	Frequency
Heterotrophic Bacteria, CFU/ml	< 10	Monthly
Resistivity, mohm-cm at 25 C	> 10	Monthly
Conductivity, mhos/cm at 25 C	< 0.1	Monthly
Total Residual Chlorine	None detected	Monthly
SiO_2, mg/L	< 0.05	Annually
Metals (Al, As, Cr, Co, Cu, Fe, Pb, Ni, Zn)	< 1 ì g/L each	Annually
Metals (Cd, Hg, Ag)	< 100 ng/L each	Annually
Total Organochlorine Pesticides and PCBs	< 50 ng/L	Annually
Total Dissolved Solids	None detected	Annually
Total Organic Carbon	< 0.05 mg/L	Annually

4. A source of compressed air free of oil, fumes and dust. Delivery through a house air system or a compressed air tank must have an in-line filter. The in-line filters must be changed regularly. Any fundamental changes in the system, as well as routine maintenance, must be documented. Diaphragm air pumps, such as aquarium pumps, are acceptable and do not require in-line filters. However, filters are strongly recommended for all air systems.

The tubing, connectors, and air stones must be made of non-toxic materials. If any part of the air

delivery system comes in contact with an effluent sample, a test solution, or other contaminant, it must be washed according to the procedures referenced in section 5.E, or it must be discarded.

5. Analytical Balance: The balance must be capable of accurately weighing 0.0001g and must be calibrated monthly using Class S, Class S-1, or ASTM 1 reference weights. Monthly calibrations must be documented. The weights used must cover the range in which the balance is normally used. The balance must be serviced at least annually by the manufacturer or a certified agent.

6. Thermometer: A thermometer traceable to National Institute of Standards and Technology (NIST) must be available and used to check all temperature monitoring devices in use for accuracy annually. Accuracy checks must be documented, and all temperature measuring devices must be labeled with the date checked, the analyst, and the correction needed. This correction value must be taken into account before temperature measurements are documented. All thermometers must be accurate to the nearest 0.1 C.

7. Vessels: Transparent or translucent vessels and non-airtight covers must be used to house culture and test organisms. Holding tanks, or vessels, for culture and dilution waters must be made of non-toxic materials. It is strongly recommended that the laboratory rinse these vessels before use.

8. Sample Containers: Sample containers of sufficient volume must be made of non-toxic materials. Sample containers must be tested for toxicity before use, and this test must be documented. Test organisms maintained in culture water which has been held in the sample containers for the maximum allowable holding time must meet the minimum performance criteria for valid controls. When the manufacturing process for the containers changes or when a new vendor is used, the test must be repeated. Plastic or disposable sample containers must not be reused. It is strongly recommended that the laboratory puncture each container after use to prevent reuse.

9. Glassware: Class A glassware (volumetric flasks, volumetric pipettes, graduated cylinders, serological pipettes, etc.) Must be used. Glass droppers or pipettes with 2 mm inside diameter and fire-polished edges must be used to transfer organisms.

10. All of the equipment necessary for the preparation of the algal diet and the YCT components and diet. This includes, but is not limited to, a microscope, hemocytometer, fluorescent light sources, drying oven (for total solids determinations), and blenders.

Laboratories should follow the recommendations in EPA/600/4-90/027F and EPA-600-4-91-002. Equipment containing copper, galvanized material, rubber, brass, and lead are not acceptable. If equipment is made of material with unknown or questionable toxicity, analysts must verify through extensive testing that it is not toxic.

C. Cleaning

New and used laboratory equipment must be washed according to the procedures outlined in EPA/600/4-90/027F or EPA-600-4-90-002. Cleaning procedures must be described in the laboratory s written procedures manual.

D. Culturing *Ceriodaphnia dubia*

C. dubia used in acute and chronic toxicity tests must come from cultures that have been started, maintained, and characterized according to the minimum requirements outlined in this section.

Laboratories must use organisms obtained directly from individual cultures for tests.

1. Initiation of Cultures

The following are the records that must be maintained and the minimum criteria that must be met for beginning in-house cultures:

a. The laboratory must document the source of the starter cultures and the date received. A laboratory`s starter cultures of Ceriodaphnia dubia must be obtained from cultures of known taxonomic identity and purity.

b. The laboratory must clearly document, in a step-by-step description, the way in which in-house cultures were begun. The offspring of a single female must be used to start cultures. She must be separated from the other organisms, and after her offspring are collected, must be sacrificed, preserved and mounted on a glass microscope slide so that the anatomical features necessary for taxonomic identification to species are observable. The slide must be dated and the initials of the taxonomist making the identification must be included.

The original starter female must be available for inspection as long as her descendants are used for testing. This includes cultures derived from this culture. If a new culture line has been started with an organism from an outside source, the previous starter female must be available for at least three years after her descendants were used for tests.

A professional taxonomist with expertise in Cladocerans must identify, or verify identification, of the laboratory's animals each time a culture line is started. The record of this must include the name and signature of the taxonomist, the taxonomic reference(s) used, and the date the organism was identified.

2. Culture Maintenance

a. Culture Water:

Animals must be cultured exclusively in synthetic water. At no time should cultures be exposed to natural or surface water. Culture water must be either moderately hard synthetic freshwater made with reagent grade chemicals or moderately hard diluted mineral water as described in section 7.3.2, page 34 of the EPA manual 600/4-90/027F. Preparation of synthetic freshwater made with Perrier must include an aeration period of at least 24 hours. Both types of synthetic waters must be made with reagent grade (Millipore Milli Q or equivalent) water (ASTM Type I water). Hybrid water prepared using both mineral water and reagent grade chemicals must not be used. Any deviation from the instructions provided in the EPA manual must be requested by the laboratory in writing and approved by this Office in writing before the change is incorporated into routine testing protocol. If there is a change in the components or the ratios of

the components, the animals must be maintained in the new culture water for at least two weeks before they can be used for toxicity tests or reference toxicity tests.

The laboratory must document the date and time prepared, date of final use, analyst, pH, conductivity, alkalinity, and hardness of each batch, or lot, of culture water.

The pH, alkalinity and hardness of synthetic freshwater made with reagent grade chemicals must be in the ranges for moderately hard water, as specified in Table 6, page 36, EPA/600/4-90/027F. The pH, alkalinity, and hardness of synthetic freshwater made using mineral water (Perrier) must be within the ranges for moderately hard water, as specified in Table 7, page 36, EPA/600/4-90/027F.

A batch of culture water must be used within fourteen (14) days after preparation.

b. Diet

C. dubia in culture and as test animals must be fed Selenastrum capricornutum and yeast-cereal leaves-trout chow (YCT) suspension.

The final algal diet suspension must be in moderately hard reconstituted water or diluted mineral water.

The analyst, date harvested, the expiration date, and the cell density (number of cells per ml) with counts and calculations must be documented. The cell density of the final diet suspension must be 3.3 to 3.5 x 10^7 cells/ml.

Algal diet suspension must be stored refrigerated and must not be used after 30 days following the date of harvest.

The date received, date opened, manufacturer, lot number, and expiration date must be documented for each component of the YCT diet.

The trout chow must meet current U. S. Fish and Wildlife specifications and must be stored frozen in a labeled moisture-proof container. A batch of trout chow can be used no longer than one year following the date manufactured.

Each batch of YCT must be tested before use and must meet the requirements for the laboratory water used for the culture/dilution water as described in Section 5.B.3. The requirements apply to the metals and total organochlorine pesticides/PCBs only.

These results must be documented and must include the date analyzed and the lot number for the YCT. A batch of YCT must not be used longer than three months after preparation, even if stored frozen. The laboratory will not have to reanalyze subsequent batches of YCT unless the lot of a component changes.

Powdered (not flake) cereal leaves must be used, the container must be stored in a desiccator, and the product can be used for no longer than two years following the date it was received.

Laboratories must use baker's yeast, available in grocery stores and from scientific supply companies. It must be stored according to package instructions. A package should not be used beyond the manufacturer's expiration date.

For each batch of YCT suspension, the total solids (in g/L), dates prepared, date frozen, expiration date, and lot numbers of the components must be documented.

Each batch of YCT must have 1.7 - 1.9 g/L solids. The frozen YCT suspension must not be used after the expiration date of any component used in its preparation. Fresh or thawed YCT suspension must be refrigerated and must not be used longer than two weeks after the date it was prepared (if not frozen) or thawed (if frozen immediately after preparation).

The laboratory must demonstrate that each batch of YCT is satisfactory by testing at least twenty (20) neonates by comparing the new batch with a batch of known quality in a side-by-side test.

Neonates fed a batch of YCT suspension must meet the minimum criteria for control survival and reproduction for a valid chronic three-brood test.

c. Propagation

An individual culture is one animal per container; a mass culture is more than one animal per container.

The documentation which is required for individual cultures is generated on a group of individual cultures. All individuals in a group must be within 12 hours age of each other, must be maintained in the same environmental chamber, and must be fed from the same batches of algal and YCT suspensions and renewed at the same time.

Documentation must be maintained for each group of individual cultures and each mass culture. The lineage of culture organisms and neonates used as test animals must be traceable back to the original female used to start the culture line. A specimen from the culture must be sacrificed, preserved, and mounted on a permanent slide every six months. This specimen must be identified to species, and the slide must be labeled with the date sacrificed, culture line identification, taxonomist, and species. Mounted specimens must be available for at least three years after the culture line from which they originate has been terminated.

For individual cultures, water must be changed at least three non-consecutive days out of seven. For mass cultures, water must be changed at least twice per week. When water is changed, the offspring of the brood animals must be removed. The dates, times and analyst changing the culture water must be documented.

Cultures must be fed six out of seven days. The YCT must have been shown to support acceptable survival and reproduction rates over a 3-brood period. The number of algal cells and the volume of YCT suspension must be consistent. Document the times and dates of feeding, the batch numbers of algal and YCT suspensions used, the volumes of the diet suspension fed to the cultures, and the analyst.

Brood animals in culture must not be more than 14 days old. Neonates used to replace brood animals must be placed in fresh, clean culture containers. Document the time and date when starting a culture with neonates, and the date when brood animals in a culture are replaced with neonates.

Neonates used to start or renew a culture must not be older than 24 hours, they must be within 12 hours age of each other, and they must not be taken from brood animals more than 14 days old. Document the age of neonates used to start or renew a mass culture or a group of individual cultures, and document the age of the brood animals from which the neonates were obtained.

One individual must be maintained in at least 15 ml of culture water in a 30 ml beaker. Any container with a similar surface area to volume ratio, and which is made of a non-toxic material may be substituted for the 30 ml beaker. New containers should be rinsed before use, especially if the laboratory suspects that chemicals used in the manufacturing process remain on the containers. Document the composition and the volume of the container in which individual cultures are maintained. While this may be presented in the Standard Operating Procedures document, if there is a change in the containers used, the change must be documented in culture logbooks.

Test animals must not be taken from a group of individual cultures if more than 20% of the original neonates are males or if more than 20% of the brood animals in the culture die, or if the average number of neonates produced in the first 3 broods is less than 15. Document the incidence of males, reproduction rate, and mortality for each group of individual cultures. Reproduction rate must be determined using at least 20% of the group.

There must be no more than 50 brood animals per liter of culture water. Document the number of neonates used to start or renew a mass culture, and the volume of the culture.

If more than 20% of the original neonates are male, or if more than 20% of the brood animals die, the mass culture must not be used as a source for test animals. Document the incidence of males and mortality.

E. Reference Toxicant Tests

Reference toxicant tests must be used to define and monitor the precision and sensitivity of the laboratory's toxicity testing system(s).

Laboratories certified for acute toxicity testing must define and measure the culture s acute response, and laboratories certified for chronic toxicity testing must define and measure the culture s chronic response.

Since the true acute and chronic toxicity responses of C. dubia have not yet been defined by the U. S. EPA, the accuracy of the tests cannot be determined. Because of this, quality control routines must be focused on achieving consistent precision and sensitivity.

1. Minimum Requirements

The following are minimum requirements for the reference toxicant tests:

a. U. S. EPA-recommended reference toxicants must be used.

b. A chemical used as a reference toxicant must be at least reagent grade. The

manufacturer's seal on the container must be broken in the laboratory. The date the chemical is received by the laboratory and opened must be written on the container and it must be stored in a desiccator. This container of reference toxicant must not be used for any other purpose but as a source for making test solutions for reference toxicant tests.

c. The tests must be definitive. To characterize the acute response, analysts must perform 48-hour static non-renewal exposures at five reference toxicant concentrations and the control, resulting in an LC50 value bounded by a 95% confidence interval. To characterize the test stock's chronic responses, the analysts must perform three-brood chronic static renewal exposures at five reference toxicant concentrations and the control.

d. Characterizing the Toxic Response

Each culture line must be characterized for its acute and/or chronic response. The following are the minimum requirements for this baseline data:

1) The cultures must be characterized for response to at least one reference toxicant.

2) A minimum of five or six reference toxicant tests must be performed. These tests must be performed over a period of time ranging from four weeks to 11 months. Each successive test must be performed within thirty days from the previous test, but on a successive generation.

3) In addition to the documentation required for effluent toxicity tests, documentation for each reference toxicant test must include the lot number of the chemical used and the concentrations of the reference toxicant, in mg/l.

4) All tests which comprise the laboratory's baseline data set must have been generated using the same reference toxicant, the same concentrations of reference toxicant, and the same batch of YCT suspension.

2. Setting Control Limits

Following initial characterization of a culture line, control limits must be defined for the acute and chronic responses.

Minimum acceptable sensitivities must be specified for the acute and chronic procedures.

The actions which will be taken when the control limits are exceeded or when the minimum sensitivity is not met must be presented in detail in the SOP document.

3. Monitoring the Toxic Response

On a monthly basis, and whenever a change is made in the system, analysts must demonstrate that the precision and sensitivity of their test system is at least as good as they achieved in the baseline reference toxicant tests.

If the toxicity responses determined by reference toxicant test do not fall within the expected ranges, as defined in the previous section, the test system and procedure must be examined and the cause of the altered toxicity values must be determined and corrected.

Laboratories are required to run a reference toxicant test under the following conditions:

a. Monthly.
b. When a new culture line is started.
c. When there is a change in culture water.
d. When equipment of unknown toxicity is used.
e. When the aeration system is changed.
f. When changes are made to the water purification system.
g. When a previous test falls outside the limits.

When a reference toxicant test result falls outside of the established limits, a second reference toxicant test must be performed within three days. If the results of the second test fall outside the acceptable range, testing must cease until the problem is identified and eliminated. A third reference toxicant test which produces results falling within the acceptable range must be performed before testing can resume. Tests for NPDES compliance purposes performed before an acceptable reference toxicant test has been conducted will be considered invalid.

F. The Sample

The laboratory performing the test must determine if the sample is valid or not valid. It is the laboratory s responsibility to inform the client of any deficiencies noticed with the sample upon receipt. It is strongly recommended that the laboratory document all communication between it and the client regarding the integrity of the sample.

G. The Test

Tests must be performed on 16-hour light/8-hour dark cycles. Ambient fluorescent light intensity is sufficient, but must not be less than 100 foot candles (ft-c). The photoperiod must be confirmed and documented every six months or whenever the timing mechanism is adjusted. The time on and time off of one complete cycle must be checked. The photoperiod must be accurate to 10 minutes.

Temperatures must be maintained at 25^{o} 1^{o} C. On each day of the test, document the temperature of the environmental control system and the test solutions.

Unless specified in the NPDES permit or required for a toxicity reduction evaluation, dilution water and culture water must be the same and must be consistent with Tables 6 or 7 in EPA/600/4-90/027F. Dilution water preparation records must be maintained and must include the date prepared, batch number, pH, conductivity, hardness, and alkalinity of the dilution water.

The conductivity, alkalinity and hardness for each new sample must be documented prior to using it in the test. The dissolved oxygen and pH must be determined and documented at the beginning and end of each 24-hour exposure period in at least one replicate in the high, medium and low test concentrations and

the control.

Chronic test solutions must be renewed daily, at plus or minus one hour of the start time of the test. The date and time of test solution renewals, the sample identification, the batch number of the dilution water, and the initials of the analyst must be documented.

A sample or test solution should be aerated only if dissolved oxygen is at less than 40% saturation, or greater than 100% saturation. Samples and solutions should be aerated for the minimum amount of time required to bring dissolved oxygen levels within these limits; every effort should be made to restrict this to five minutes. Document aeration of the sample prior to the test and aeration of the test solutions. Documentation must include the length of time the sample or solution is aerated and the dissolved oxygen levels before and after aeration.

It may be necessary to first coarse-filter samples through a sieve having 2- to 4-mm mesh openings to remove debris and/or break up large floating or suspended solids. If samples contain indigenous organisms that may attack or be confused with the test organisms, the samples must be filtered through a sieve with 60 m mesh openings. Be aware that filtering may remove some toxicity. The laboratory must document filtration of samples.

Should a test fail, a general description of the sample(s) may be helpful in interpreting the result. Even samples that are not unusual must be described. Certainly, anything unusual about the sample that might hint at underlying causes of potential toxicity must be recorded. Examples would be a heavy precipitate, high turbidity, presence of organisms that might prey on C. dubia, an odor, immiscible liquids, color, heterogeneous viscosity in the sample, long-lasting foam or bubbles, or other characteristics or changes. Document the general appearance of each sample with respect to clarity, odor, color and other noticeable characteristics.

1. Test Animals

Test animals in acute and chronic tests must be taken from cultures for which there is sufficient documentation to prove the cultures are taxonomically pure. Organisms must be taken from cultures on which there are current reference toxicant test data. The reference toxicant tests must have been performed no more than thirty (30)days before the animals are used to test an effluent sample. Organisms must be from cultures with at least 90 percent survival and less than 10 percent males. Also, 20 percent of the organisms, chosen at random, must produce an average of 15 neonates in the first three broods. Neonates used in tests must come from mothers which are 14 days old or less. Neonates must be less than 24 hours old, and those used in chronic tests must have been hatched within 8 hours of each other. The laboratory must have a system in place which will clearly demonstrate that these neonates are of the required age. The neonates used in tests cannot be from a first or second brood.

To prevent changing the effluent concentration significantly, the animals must be transferred in the least possible volume, not to exceed 1% of the test solution volume (0.15 ml for 15 ml solution in a 30 ml medicine cup). The maximum volume required to transfer neonates must be determined for each analyst at least once every six months. Documentation of these checks must be maintained.

The animals must be fed once daily, at the time test solutions are renewed, or immediately after this. The combined volumes of YCT and algal diet suspensions added to a test vessel must not be more

than 0.2 ml (a ratio of 0.2 ml diet/15 ml test solution must not be exceeded). The volumes of diet, and number of algal cells added to the test vessels must be consistent throughout the test. The same batch of YCT suspension must be used throughout a test, and the YCT must have been shown to support acceptable survival and reproduction rates over a 3-brood period. These feedings must be documented. The information must include the volumes of algal and YCT diet suspensions added to each replicate, the batch numbers of YCT diet and algal suspensions, the times the animals are fed and the analyst.

2. Test Validity

The following records must be kept and standards met to demonstrate that a test is valid.

a. The date and time a test was started and terminated. An acute test must run for 48 hours. A chronic test must not be terminated until at least 60 percent of the control animals have had their third brood. If at least 60 percent of the control animals have had their third brood before the completion of day 5, the test must continue for a sixth day. At a minimum, the organisms in a chronic test must be exposed to each sample in the series a minimum of 48 hours.

b. Document the survival of the control animals. For a valid chronic test, the mortality of the control animals must not exceed 20%. For a valid acute test, mortality of the control animals must not exceed 10%.

c. For chronic tests, document the incidence of males. The incidence of males must not exceed 20 percent.

NOTE: The mortality and incidence of males combined must not exceed twenty percent in the chronic test.

For a Pass/Fail Chronic test, a minimum of 20 replicates in the control and 20 replicates in the effluent treatment are necessary for a valid comparison of reproduction. For definitive chronic tests, at least six replicates in the control and at least six replicates in each effluent treatment that does not produce significant mortality are necessary to make a valid estimate of the NOEC and the LOEC.

A chronic test is not valid unless the average number of young produced per female in three broods is at least fifteen (15). For the control animals in the chronic tests, document the average total number of young produced per female in three broods.

The early termination of a valid test for no apparent reason may result in criminal charges. Regulation 61-9, Water Pollution Control Permits, page 72, paragraph (5) states The Clean Water Act provides that any person who falsifies, tampers with, or knowingly renders inaccurate any monitoring device or method required to be maintained under this permit shall, upon conviction, be punished by a fine of not more than $10,000 or by imprisonment for not more than two years, or both. If a conviction of a person is for a violation committed after a first conviction of such person under this paragraph, punishment is a fine of not more than $20,000 per day of violation, or by imprisonment of not more than four years, or both. The reason for terminating a test before completion and resampling must be thoroughly documented.

H. Statistical Analyses

1. Analysis of Mortality Data - Acute and Chronic Pass/Fail

Mortality data from pass/fail tests are analyzed by testing the null hypothesis that test group mortality equals control group mortality versus the alternative hypothesis that test mortality is greater than control mortality. The null hypothesis is tested using a one-tailed Fisher's exact test. The test is performed only if the proportion of surviving control organisms is greater than the proportion of surviving test organisms. Otherwise, the null hypothesis is accepted and the test passes. The criterion for rejecting the null hypothesis is set by the NPDES permit or consent order in which the test is required.

Hand calculation

Construct a 2x2 contingency table using the following pattern:

Group	Live	Dead	Total
Control	*LC*	*DC*	*C*
Test	*LT*	*DT*	*T*
Total	*L*	*D*	*N*

where *LC* is number of live control organisms, *LT* is number of live test organisms, *DC* is number of dead control organisms, *DT* is number of dead test organisms, *C* is *LC*+*DC*, etc., and *N* is the total number of organisms used in the test. The *P* value from Fisher's exact test (P_{FET}) is

$$P_{FET} = \frac{\frac{\Sigma C!\,\Sigma T!\,\Sigma L!\,\Sigma D!}{N!}}{LC!\,DC!\,LT!\,DT!} \qquad (1)$$

Computational Algorithm

This section shows the derivation and general form for an algorithm for computing P_{FET}. The next section shows an example of the computation. The reader may want to read the next section first, and then read this section for more information and for programming puroses.

The arithmetic can be simplified by cancelling some factors before doing the multiplication. *LC*, *LT*, *DC*, and *DT* are cells, and *C*, *T*, *L* and *D* are marginal totals. To simplify, arrange the marginal totals and cells in equation (1) in order of descending value. Equation (1) can be rewritten

$$P_{FET} = \frac{\frac{T_4!\,T_3!\,T_2\,T_1}{N!}}{C_4!\,C_3!\,C_2!\,C_1!} \qquad (2)$$

where T_4 is the largest marginal total value and C_4 is the largest cell value. 0!'s and 1!'s can be ommitted. Let $I = \frac{T_4!}{N!}$, and cancel factors which appear in both the numerator and denominator before multiplying the

remaining ones. Then equation (2) becomes

$$P_{FET} = \frac{T_3!\,T_2!\,T_1!}{C_4!\,C_3!\,C_2!\,C_1!} * I \qquad (3)$$

Let $J = \frac{T_3!}{C_4!}$, and then equation (3) becomes

$$P_{FET} = \frac{T_2!\,T_1!}{C_3!\,C_2!\,C_1!} * I * J$$

Repeat the process until equation (2) becomes

$$P_{FET} = \frac{1}{C_1!} * I * J * K * L$$

which, in cases where P_{FET} is large, can be evaluated on a calculator with a minimum of data entry.

Numerical Examples

EXAMPLE 1. An acute test using 40 organisms resulted in 20 live control organisms, 0 dead control organisms, 18 live test organisms and 2 dead test organisms. Putting this data in a contingency table gives

Group	Live	Dead	Total
Control	20	0	20
Test	18	2	20
Total	38	2	40

At this point it is necessary to introduce product notation. $\prod_{i=j}^{n} i$, where *n* and *j* are integers and *n*>*j*, means to multiply the values of *i*, the first being *j* and the last being *n*. It is also important to note that 0! is defined to be 1. Arranging the cells and totals for computation using equation (2) gives $P_{FET} = \frac{\frac{38!\,20!\,20!\,2!}{40!}}{20!\,18!\,2!}$

Now, $I = \frac{T_4!}{N!}$ so $I = \frac{38!}{40!} = \frac{1}{\prod_{i=39}^{40} i} = \frac{1}{39*40}$ since all integers from 1 to 38 appear as factors in both the numerator and denominator. Then, $P_{FET} = \frac{20!\,20!\,2!}{20!\,18!\,2!} * \frac{1}{39*40}$ Dropping factors which appear in both the

numerator and denominator leaves $P_{FET} = \frac{20!}{18!} * \frac{1}{39*40}$. Since $\frac{20!}{18!} = \prod_{i=19}^{20} i = 19*20$,

$P_{FET} = 19*20*\frac{1}{39*40} = 0.2436$. Since this *P* value is greater than 0.05, this data is not statistically significant evidence that the proportion of live test organisms is less than the proportion of live control organisms, so the test is a pass.

EXAMPLE 2. An acute toxicity test using 40 organisms resulted in 19 live control organisms, 1 dead control organism, 12 live test organisms and 7 dead test organisms. One test organism was lost. The contingency table is

Group	Live	Dead	Total
Control	19	1	20
Test	12	7	19
Total	31	8	39

Arranging for computation gives $P_{FET} = \frac{\frac{31!20!19!8!}{39!}}{19!12!7!1!}$, so $I = \frac{T_4!}{N!} = \frac{31!}{39!} = \frac{1}{(\prod_{i=32}^{40})}$ and

$P_{FET} = \frac{20!19!8!}{19!12!7!1!} * \frac{1}{(\prod_{i=32}^{39})}$. Dropping a one which is only a factor and continuing gives

$$P_{FET} = 20*\prod_{i=13}^{19} i*8*\frac{1}{\prod_{i=32}^{39} i} = 0.0164 .$$

Base SAS

Fisher's exact test can be perfomed by the Base SAS procedure FREQ. The following statements will analyze the dataset used in the example above.

```
proc format;  /* Define formats for more readable output*/
    value grname 1='Control group' 2='Test group';
    value rtype 1='Live' 2='Dead';

data acute;  /*create set of acute pass/fail data*/
    format group grname. response rtype.; /*associate variable 'group' with format 'grname.' and var
    'response' with format 'rtype.'*/
    do group=1 to 2;/*1=control, 2=test*/
```

```
        do response=1 to 2;/*1=live, 2=dead*/
            input counts @@;/* trailing @@ allows all input values to go on one line*/
            output;/*write values to data set*/
        end;/* close iterative do loop response=1 to 2*/
    end;/*close iterative do loop group=1 to 2*/
    cards; /*data on next line. Control live, control dead, test live, test dead*/
20 0 18 2
;
/*analyze contingency table using PROC FREQ, and BASE SAS product*/
proc freq data=acute;
    weight counts; /*counts is number in each cross-classification*/
    tables group*response/exact; /*define cross-classification and request Fisher's exact test*/
run; quit;
```

The order the values are listed after the CARDS statement is important. In this example, the order is number of live control, dead control, live test and dead test organisms. SAS will report a left-tailed, a right-tailed and a two-tailed *P*value. When this order is used, the right-tailed *P* value is the one of interest. SAS output for this example

TABLE OF GROUP BY RESPONSE

GROUP	FREQUENCY		
Frequency Percent Row Pct Col Pct	Live	Dead	Total
Control Group	20 50.00 100.00 52.63	0 0.00 0.00 0.00	20 50.00
Test Group	18 45.00 90.00 47.37	2 5.00 10.00 100.00	20 50.00
Total	38 95.00	2 5.00	40 100.00

STATISTICS FOR TABLE OF GROUP BY RESPONSE

Statistic	DF	Value	Prob
Chi-Square	1	2.105	0.147
Likelihood Ratio Chi-Square	1	2.878	0.090
Continuity Adj. Chi-Square	1	0.526	0.468
Mantel-Haenszel Chi-Square	1	2.053	0.152

Fisher's Exact Test (Left)	1.000
(Right)	0.244
(2-Tail)	0.487
Phi Coefficient	0.229
Contingency Coefficient	0.224
Cramer's V	0.229

Sample Size = 40
WARNING: 50% of the cells have expected counts less than 5. Chi-Square may not be a valid test.

2. Analysis of Reproduction Data

Reproduction data from pass/fail chronic tests are analyzed according to USEPA (1994) Appendix H, with one modification. USEPA (1994) describes the equal-variance Shapiro-Wilk's test, in which the data are centered on their group means before being pooled and analyzed as one sample. Because it is not reasonable to assume that group variances will be equal (this assumption is tested if the data are normally distributed via the *F* test), Shapiro-Wilk's test is perfomed on both groups separately, using raw data. Paragraph 2.3 in USEPA (1994) Appendix B is skipped, and *W* and a *P* value is computed for both groups. If either group is non-normal, the null hypothesis of no effect is tested with Wilcoxon's Rank Sum or the Mann-Whitney *U* test. If both groups are normally distributed, the assumption of homogeneity of variance between groups is tested with an *F* test. If this assumption is met, Student's *t* test is used to test the null hypothesis of no effect. If the group variances are heterogeneous, the null hypothesis is tested with Satterthwaite's *t*.

A test is analyzed only if test group average reproduction is less than control group average reproduction. Otherwise, the test is a pass. The rejection criterion for the final hypothesis test (Student's or Satterthwaite's *t*, or Wilcoxon's Rank Sum) is specified in the NPDES permit or consent order.

Numerical Examples

Two examples of chronic data are provided to demonstrate the use of SAS and allow other software or procedures to be checked. Although the final hypothesis test used is conditional on the outcome of the assumption tests, these data sets can be used to check all quantities that may computed. That is, even though both groups in the first example are normally distributed, the data can still be used to check computation of Wilcoxon's *W* and P_W.

EXAMPLE 1 DATA.

Control	17	21	28	18	26	18	13	20	22	17	19	16	25	14	16	24	24	23	28	25
Test	13	11	19	13	18	10	19	11	17	16	16	26	10	19	20	23	15	14	21	13

EXAMPLE 1 RESULTS.

	Group			
	Control	Test		
Size	20	20	F	1.05
Average	20.7	16.2	P_F	0.9154
Variance	20.74737	19.74737	Student's t	3.1625
Sum of squares	394.2	375.2	d.f.	38
Shapiro-Wilk's W	0.955912	0.959264	P_t	0.0031
P_W	0.47437	0.53489	Satterthwaite's t	3.1625
			d.f.	38
			P_t	0.0031
			Wilcoxon's W	511.5
			P_W	0.0062

EXAMPLE 1 DISCUSSION.

Both groups are normally distributed (control P_W=0.47437, test P_W=0.53489), and the variances are equal (P_F=0.9154), so Student's t test is used, and the test is a fail at the 0.05 á level (P_t=0.0031).

EXAMPLE 2 DATA.

Control	20	12	10	10	12	22	21	20	23	18	25	7	20	5	14	27	28	16	35	11
Test	10	14	8	8	7	11	9	1	13	14	8	20	20	17	14	19	23	19	13	8

EXAMPLE 2 RESULTS.

	Group			
	Control	Test		
Size	20	20	F	1.91
Average	17.8	12.8	P_F	0.1684
Variance	59.95789	31.43158	Student's t	2.3390
Sum of squares	1139.2	597.2	d.f.	38
Shapiro-Wilk's W	0.97472	0.959547	P_t	0.0247
P_W	0.83510	0.54019	Satterthwaite's t	2.3390
			d.f.	34.6
			P_t	0.0252
			Wilcoxon's W	487
			P_W	0.0381

EXAMPLE 2 DISCUSSION.

Both groups are normally distributed (control P_W=0.83510, test P_W=0.54019) and variances are homogeneous (P_F=0.1684), so Student's t test is used. The test is a fail at the 0.05 á level (P_t=0.0247).

SAS/STAT

The following code for SAS/STAT will perform the analyses described above.

```
/*Produce a title for the ouput*/
title1 'SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example';

proc format;  /* Define formats for more readable output*/
    value grname 1='Control group' 2='Test group';

/* create set of chronic pass/fail test data*/
data chronic;
    format group grname.; /*Associate variable 'group' with format 'grname.'*/
    input group young;
    cards;/*Data starts on next line*/
1 17 /*Control group data.  Group #, # young*/
1 21
1 28
1 18
1 26
{Enter rest of data for control group.  Erase this line}

2 13 /*Test group data.  Group #, # young*/
2 11
2 19
2 13
2 18
{Enter rest of data for test group.  Erase this line}
;

/*Shapiro-Wilk's using PROC UNIVARIATE, a BASE SAS product*/
proc univariate data=chronic normal; /*normal requests SW*/
  var young;  /*Analyze variable young*/
  by group;  /*Perform analysis on variable young grouped by variable group*/

/*F, Student's and Satterthwaite's t tests.  PROC TTEST is in SAS/STAT*/
proc ttest data=chronic;
  class group;  /*Data are classified by values of variable group*/
  var young;  /*Analyze variable young*/

/*Wilcoxon's test.  PROC NPAR1WAY is in SAS/STAT*/
proc npar1way data=chronic wilcoxon; /*Request use of wilcoxon's test*/
  class group; /*Data are classified by values of variable group*/
  var young; /*Analyze variable young*/

run; /*execute all statements since last proc or data step*/
quit; /*quit any data or proc steps still open*/
```

The following SAS output was produced by the numerical examples and code from above. The *P* value for Shapiro-Wilk's test is listed after *Pr<W* in the SAS output for PROC UNIVARIATE. A report is produced for each group. The *P* values for the *F*, Student's *t* and Satterthwaite's *t* tests are in the PROC TTEST output. The *P* value for the *F* test is reported after *Prob>F´* =. The *P* values for the *t* tests are under a column headed *Prob> T* . Since the reported *P* values are two-tailed, they must be divided by two to make them one-tailed. The *P* value for Wilcoxon's test is listed in the output for PROC NPAR1WAY after *Prob > Z* =, under the *Wilcoxon 2-Sample Test* section.

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 1

---------- GROUP=Control group ----------

Univariate Procedure

Variable=YOUNG

Moments				Quantiles(Def=5)			
N	20	Sum Wgts	20	100% Max	28	99%	28
Mean	20.7	Sum	414	75% Q3	24.5	95%	28
Std Dev	4.554928	Variance	20.74737	50% Med	20.5	90%	27
Skewness	0.037946	Kurtosis	-1.1198	25% Q1	17	10%	15
USS	8964	CSS	394.2	0% Min	13	5%	13.5
CV	22.00448	Std Mean	1.018513			1%	13
T:Mean=0	20.32375	Pr>|T|	2.E-14	Range	15		
Num ^= 0	20	Num > 0	20	Q3-Q1	7.5		
M(Sign)	10	Pr>=|M|	2.E-06	Mode	16		
Sgn Rank	105	Pr>=|S|	2.E-06				
W:Normal	0.955912	Pr<W	.47437				

Extremes

Lowest	Obs	Highest	Obs
13(	7)	25(	13)
14(	14)	25(	20)
16(	15)	26(	5)
16(	12)	28(	3)
17(	10)	28(	19)

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 1

---------- GROUP=Test Group ----------

Univariate Procedure

Variable=YOUNG

Moments				Quantiles(Def=5)			
N	20	Sum Wgts	20	100% Max	26	99%	26
Mean	16.2	Sum	324	75% Q3	19	95%	24.5
Std Dev	4.443801	Variance	19.74737	50% Med	16	90%	22
Skewness	0.416318	Kurtosis	-0.37995	25% Q1	13	10%	10.5
USS	5624	CSS	375.2	0% Min	10	5%	10
CV	27.43087	Std Mean	0.993664			1%	10
T:Mean=0	16.3033	Pr>|T|	1.E-12	Range	16		
Num ^= 0	20	Num > 0	20	Q3-Q1	6		
M(Sign)	10	Pr>=|M|	2.E-06	Mode	13		
Sgn Rank	105	Pr>=|S|	2.E-06				
W:Normal	0.959264	Pr<W	.53489				

Extremes

Lowest	Obs	Highest	Obs
10(	13)	19(	14)
10(	6)	20(	15)
11(	8)	21(	19)
11(	2)	23(	16)
13(	20)	26(	12)

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 1

TTEST PROCEDURE

Variable: YOUNG

GROUP	N	Mean	Std Dev	Std Error	Minimum	Maximum
Control group	20	20.70000000	4.55492793	1.01851285	13.00000000	28.000000
Test group	20	16.20000000	4.44380112	0.99366414	10.00000000	26.000000

Variances	T	DF	Prob>|T|
Unequal	3.1625	38.0	0.0031
Equal	3.1625	38.0	0.0031

For H0: Variances are equal, F' = 1.05 DF = (19,19) Prob>F' = 0.9154

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 1

N P A R 1 W A Y P R O C E D U R E

Wilcoxon Scores (Rank Sums) for Variable YOUNG
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
Control	20	511.500000	410.0	36.8851301	25.5750000
Test gro	20	308.500000	410.0	36.8851301	15.4250000

Average Scores Were Used for Ties

Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S = 511.500 Z = 2.73823 Prob > |Z| = 0.0062

T-Test Approx. Significance = 0.0093

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ = 7.5723 DF = 1 Prob > CHISQ = 0.0059

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 2

---------- GROUP=Control ----------

Univariate Procedure

Variable=YOUNG

Moments

N	20	Sum Wgts	20
Mean	17.8	Sum	356
Std Dev	7.743248	Variance	59.95789
Skewness	0.316121	Kurtosis	-0.28616
USS	7476	CSS	1139.2
CV	43.5014	Std Mean	1.731443
T:Mean=0	10.28044	Pr>|T|	3.E-09
Num ^= 0	20	Num > 0	20
M(Sign)	10	Pr>=|M|	2.E-06
Sgn Rank	105	Pr>=|S|	2.E-06
W:Normal	0.97472	Pr<W	.83510

Quantiles(Def=5)

100% Max	35	99%	35
75% Q3	22.5	95%	31.5
50% Med	19	90%	27.5
25% Q1	11.5	10%	8.5
0% Min	5	5%	6
		1%	5
Range	30		
Q3-Q1	11		
Mode	20		

Extremes

Lowest	Obs	Highest	Obs
5(	14)	23(	9)
7(	12)	25(	11)
10(	4)	27(	16)
10(	3)	28(	17)
11(	20)	35(	19)

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 2

---------- GROUP=Test ----------

Univariate Procedure

Variable=YOUNG

Moments

N	20	Sum Wgts	20
Mean	12.8	Sum	256
Std Dev	5.606387	Variance	31.43158
Skewness	0.000159	Kurtosis	-0.43925
USS	3874	CSS	597.2
CV	43.7999	Std Mean	1.253626
T:Mean=0	10.21038	Pr>|T|	4.E-09
Num ^= 0	20	Num > 0	20
M(Sign)	10	Pr>=|M|	2.E-06
Sgn Rank	105	Pr>=|S|	2.E-06
W:Normal	0.959547	Pr<W	.54019

Quantiles(Def=5)

100% Max	23	99%	23
75% Q3	18	95%	21.5
50% Med	13	90%	20
25% Q1	8	10%	7.5
0% Min	1	5%	4
		1%	1
Range	22		
Q3-Q1	10		
Mode	8		

Extremes

Lowest	Obs	Highest	Obs
1(	8)	19(	16)
7(	5)	19(	18)
8(	20)	20(	12)
8(	11)	20(	13)
8(	4)	23(	17)

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 2

TTEST PROCEDURE

Variable: YOUNG

GROUP	N	Mean	Std Dev	Std Error	Variances	T	DF	Prob>|T|
Control	20	17.80000000	7.74324833	1.73144296	Unequal	2.3390	34.6	0.0252
Test	20	12.80000000	5.60638733	1.25362632	Equal	2.3390	38.0	0.0247

For H0: Variances are equal, F' = 1.91 DF = (19,19) Prob>F' = 0.1684

SAS Output for SCDHEC Ceriodaphnia Reproduction Data Example 2

N P A R 1 W A Y P R O C E D U R E

Wilcoxon Scores (Rank Sums) for Variable YOUNG
Classified by Variable GROUP

GROUP	N	Sum of Scores	Expected Under H0	Std Dev Under H0	Mean Score
Control	20	487.0	410.0	36.8816541	24.3500000
Test	20	333.0	410.0	36.8816541	16.6500000

Average Scores Were Used for Ties

Wilcoxon 2-Sample Test (Normal Approximation)
(with Continuity Correction of .5)

S = 487.000 Z = 2.07420 Prob > |Z| = 0.0381

T-Test Approx. Significance = 0.0447

Kruskal-Wallis Test (Chi-Square Approximation)
CHISQ = 4.3587 DF = 1 Prob > CHISQ = 0.0368